COMMUNICATION

Resistance to the "Last Resort" Antibiotic Colistin: A Single-Zinc Mechanism for Phosphointermediate Formation in MCR Enzymes

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MCR (mobile colistin resistance) enzymes catalyse phosphoethanolamine (PEA) addition to bacterial lipid A, threatening the "last-resort" antibiotic colistin. Molecular dynamics and density functional theory simulations indicate that monozinc MCR supports PEA transfer to the Thr285 acceptor, positioning MCR as a mono- rather than multinuclear member of the alkaline phosphatase superfamily.

Multidrug-resistant Gram-negative bacteria (GNB, e.g. *Escherichia coli*, *Klebsiella pneumoniae*), are a serious, growing, global public health threat.[1] Treatment options are limited by a lack of new antimicrobials[2] and dissemination of resistance plasmids.[3] The polymyxin colistin is a 'last-resort' antibiotic for GNB resistant to preferred agents (cephalosporins, carbapenems)[4] but is threatened by plasmid-encoded MCR (mobile colistin resistance) enzymes.[5] MCR expression in GNB carrying other resistance determinants (extended-spectrum or carbapenem-hydrolysing β-lactamases[6]), threatens untreatable bacterial infections and a return to the pre-antibiotic era.

MCR enzymes transfer positively charged phospho-ethanolamine (PEA) onto the 1' or 4' phosphates of lipid A (Figure S1) in outer membrane lipopolysaccharide(LPS) of GNB. PEA addition decreases lipid A net negative charge, reducing affinity for positively charged colistin. PEA transferases such as

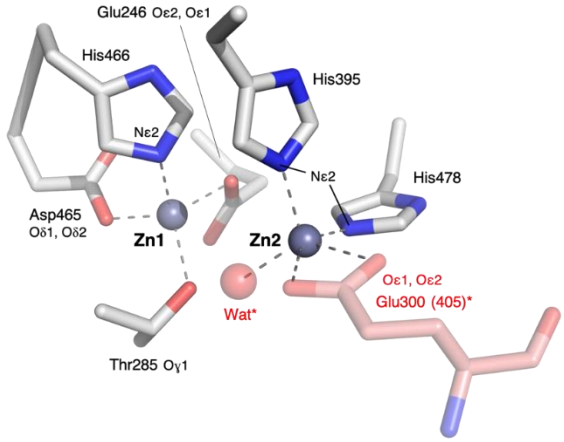

Figure 1: MCR Active Site. Zn1 and Zn2 ligating atoms in dizinc MCR-1 (PDB 5LRM) are shown. Active site residues are conserved in MCR-1 and -2 (PDBs 5LRN[7], 5LRM[7], 6SUT, 5MX9[8]) with catalytic Thr285 phosphorylated in monozinc structures (5LRN, 6SUT, see Figure S2). Additional Zn2 coordination by Glu300/405 (red) arises from contact with a symmetry related molecule.

MCR-1 are metal-dependent, inner-membrane bound enzymes, with a periplasmic catalytic domain and a conserved threonine (MCR-1 Thr285), as likely PEA acceptor in the transfer reaction.

We recently crystallised the periplasmic domains of MCR-1 and MCR-2, revealing a conserved active site resembling other bacterial PEA transferases and alkaline phosphatase (AP),[7,8] and identifying MCR proteins as members of the AP core superfamily. Our structures have either one (mono-) or two (dizinc) active site zinc ions; further structures crystallised under higher (non-physiological) zinc concentrations feature active sites with up to 5 zinc ions.[9,10] A full-length homologue (*Neisseria meningitidis* EptA, 36% identical to MCR-2) contains a single zinc ion.[11] Only one zinc site (Zn1), tetrahedrally coordinated by MCR Glu246, His466, Asp465 and Thr285 (Figure 1; Table S1) is conserved in all PEA transferase structures.[7]

Where present the second (Zn2) site is coordinated by residues His395 and His478, a water molecule, and Glu300 or Glu405 from a crystal contact with an adjacent chain (Figure 1, PDB 5LRM).[7] although the MCR-1[7] and -2[8] catalytic domains exist as monomers in solution. Furthermore, in most (8/11)

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MCR-1 and -2 structures the catalytic Thr285 is phosphorylated, possibly representing the binding mode of phospho-linked PEA substrates. In phosphorylated structures, most of which contain a single zinc ion, the Zn2-binding His395 forms a weak (~3.1 Å) hydrogen bond to phosphoThr285. Mutation to alanine of any Zn1-coordinating residues, or of His395, completely abolishes MCR-1/-2 activity, as measured by colistin susceptibility of MCR-expressing *E. coli*.[12] However, mutation of the Zn2-coordinating His478 reduced but did not abolish activity,[7] indicating this residue as important, but not essential, to activity and challenging the essentiality of an intact Zn2 site.

The mechanism of MCR-catalysed PEA transfer to lipid A is currently unknown. Resemblance to AP has motivated proposals of similar mechanisms,[9] although current models for AP catalysis involve two or three zinc ions.[13-16] Importantly, however, the AP superfamily also contains monometallic enzymes (e.g. phosphonate monoester hydrolase, PMH), where basic amino acids functionally substitute for additional metal ions to orient substrate for nucleophilic attack, stabilise the transition state and activate incoming water to degrade the covalent phospho-intermediate.[17-19] Key questions in understanding MCR enzymes then concern the number of metal ions required for the two reaction steps (PEA transfer to MCR Thr285 and subsequently to lipid A). Here we combine X-ray crystallography, molecular dynamics (MD) simulations and DFT calculations to investigate the structure, stability, dynamics and PEA transfer activity of the MCR metal centre.[20]

Crystal structures of MCR-1 and -2 in both phosphorylated (monozinc, PDB 5LRN[7], 6SUT (this work, Figure S2, Table S2)) and dephosphorylated (dizinc, PDB 5LRM[7], 5MX9[8]) states were used as starting points for molecular dynamics (MD) simulations of ≥ 200 ns. These confirmed the isolated catalytic domain as a rigid, stable entity, supporting its use as a model system for computational investigations of MCR dynamics/mechanism (Figures S3, S4). Interestingly, residues 348 – 365 show similar mobility to other solvent-exposed regions, inconsistent with previous proposals implicating this loop in substrate binding[21].

We next analysed the dynamic behaviour of Zn1 and Zn2 in MD simulations of mono- and bi-metallic MCR-1/-2, modelling the metal ions with a nonbonded Lennard-Jones 12-6 approach with restraints during minimisation, heating, and equilibration. This represents a compromise between preventing repulsion between metal ions in multinuclear systems and allowing for ligand exchange (not possible in fully bonded/dummy atom approaches) and evaluation of stability of different metallated states. In 3/5 200 - 300 ns simulations of dizinc MCR-1, and 2/3 simulations of dizinc MCR-2, zinc dissociated from the Zn2 site (His395/His478, Figure 2). Even when complete dissociation was not observed, the Zn2 RMSF (MCR-1 9.9 Å, MCR-2 4.9 Å) was consistently higher than for Zn1 (MCR-1 5.0 Å, MCR-2 3.1 Å), confirming the Zn1 site as significantly more stable than Zn2 with a likely much lower dissociation constant. Coordination distances (Figure S5) indicate only minor perturbation of the Zn1 site upon loss of Zn2. Consistent with biochemical data[22] and the *N. meningitidis* EptA structure[11], these data identify only one stable zinc site (Zn1) in the MCR catalytic domain.

Coordination geometry at the Zn1 site was then considered.

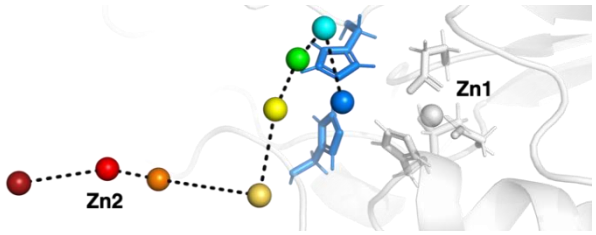

Figure 2: Dissociation of Zn2 from Unphosphorylated MCR-1. Zn2 (labelled) dissociates from MCR-1 (PDB 5LRM) after 161 ns of a 300 ns MD trajectory. Figure shows 7 snapshots spanning 161 to 161.7 ns of the trajectory, with Zn2 ion positions coloured by frame from blue (161 ns) to deep red (161.7 ns).

During MD simulations of both mono- and dizinc MCR-1/-2 the Zn1-coordinating residues Glu246, Asp465, His466 and Thr285 show significant sidechain flexibility. In monozinc MCR-1/-2, each adopts multiple distinct conformations (Figure S6); His466 moves away from the zinc ion and both carboxylate ligands (Glu246, Asp465) adopt either mono- or bidentate coordination (Figure S7, Table S3), but with minimal preference between rotamers. In simulations of the dizinc enzymes Glu246 favours mono- over bidentate Zn1 coordination, despite potential loss of a stabilising "second shell" interaction with His395, which instead coordinates Zn2. Monodentate Zn1 coordination by Glu246 is more consistent with crystal structures of both mono- and dizinc MCR-1/-2.[7, 8] While, at this level of sampling, conclusions drawn from relative rotamer preferences may be limited, these simulations both replicate crystallographically observed zinc coordination and identify active site flexibility (Asp465 and Glu246 in exchange between mono- and bidentate coordination) that may be mechanistically relevant.

MCR is expected to catalyse PEA transfer *via* a covalent, Thr285-bound phospho-intermediate, thus it is also relevant to consider how modification of Thr285 (that in unphosphorylated MCR enzymes coordinates Zn1) affects the active site. During simulations of dizinc MCR-1/-2 Thr285 Oγ maintains contact with Zn1 before and after Zn2 dissociation, indicating that unphosphorylated Thr285 stably coordinates Zn1 regardless of occupation of the Zn2 site. As above, Zn1 remains bound in the active site throughout, flexibly coordinated by Asp465 and Glu246 (Figure S8). Thus Thr285 dephosphorylation in monozinc MCR-1/-2 has limited effect on Zn1 architecture and dynamics.

Thr285 is consistently observed phosphorylated in crystal structures of monozinc MCR-1/-2, making possible additional interactions, that persist throughout MD simulations, of Zn1 with the more strongly charged terminal phosphate oxygen atom. As crystal structures of dizinc phosphorylated MCR-1/-2 remain unavailable, an additional model was created to probe the effect of Thr285 phosphorylation on stability of the Zn2 site. In MD simulations zinc remains stably bound in the Zn1 site after *in silico* phosphorylation of dizinc MCR-1/-2, with Glu246 and Asp465 in exchange between mono- and bidentate coordination, but coordination by His466 is lost and phosphoThr285 moves closer towards the Zn2 site and becomes more solvent-exposed. As expected, the proximity of the negatively-charged Thr285 phosphate group invariably

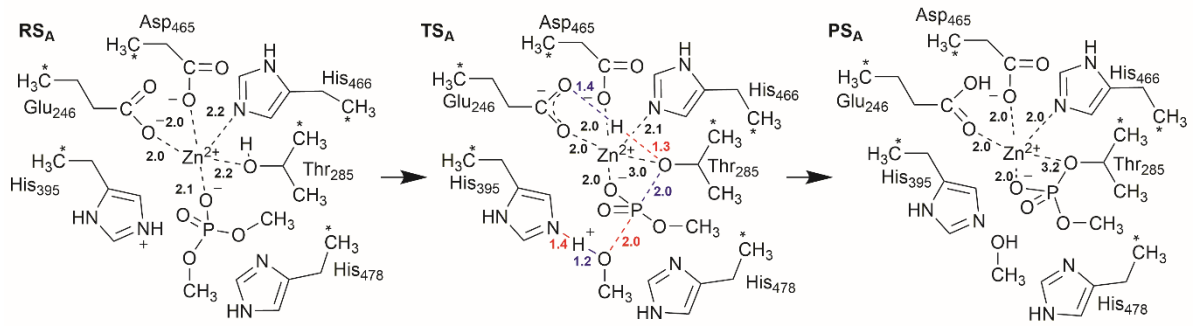

Figure 3: Favoured Reaction Path for Phosphoethanolamine (PEA) addition to MCR-1/2 Thr285. Figure shows reactant (RS), transition (TS) and product (PS) states for pathway A (favoured; pathway B shown in Figure S11), derived from DFT models for PEA transfer to Thr285 of monozinc MCR-1. PEA is represented as dimethyl phosphate (see Methods). Blue dashed lines represent bonds forming; red, bonds breaking. Asterisks (*) mark Cα atoms.

prevented complete dissociation of the second zinc equivalent, but coordination by both His395 and His478 is lost and the Zn2 ion is instead exclusively bound by two phosphate oxygen atoms in bidentate coordination.

Histidines 395 and 478 in MCR-1/-2 are both highly conserved and functionally important, as adjudged by increased colistin susceptibility when either is mutated.[7] Aside from their possible Zn2 coordination, these residues could participate in catalysis, for example stabilising/orienting incoming PEA and/or lipid A substrates *via* interactions with phosphate oxygen atoms. However, the requirements for these roles differ in that metal coordination would require deprotonation of His Nε2 atoms, whereas interaction with substrate phosphate would require their protonation. Accordingly, simulations were carried out on monozinc, dephosphorylated MCR-1 with His395 and His478 both neutral (i.e. singly protonated at Nδ) and protonated (at both Nδ and Nε) to assess the effect of protonation upon MCR active site structure and dynamics. These identify His478 as adopting a stable conformation independent of protonation at Nε, while protonation affects the conformational distribution of His395, which then favours a rotamer distinct from the crystal structures (Figures S9, S10).

The lability of the MCR Zn2 site supports the feasibility of mechanistic hypotheses that require a single zinc ion only. The initial step in MCR-catalysed lipid A modification involves PEA transfer from the membrane-embedded donor (phosphatidylethanolamine) to Thr285, likely via nucleophilic attack of Thr285 on the substrate phosphorous centre. In our previous report[8] we presented preliminary density functional theory (DFT) calculations designed to investigate the feasibility of PEA transfer by mono- and dizinc MCR-1. We now extend these experiments to a higher level of theory in order to obtain more realistic energy barriers and test mechanisms involving (previously omitted) His395 and His478. Models of the MCR-1 active site were used as starting points for DFT calculations to identify energetically feasible routes for PEA addition to Thr285.

Two candidate paths (A and B) for the reaction catalysed by monozinc MCR-1 were identified using the B3LYP functional with Grimme's dispersion correction and a combination of different basis sets. Reactants, transition states and products were identified (RS, TS and PS, respectively, Figures 3, S11), and their nature confirmed with frequency calculations. One of these pathways (B) consists of a single step where phosphate cleavage takes place simultaneously with proton transfer from Thr285 to the leaving group (dephosphorylated membrane lipid), shuttled by the transient phosphoryl group (TSB, Figure S11). In this model all histidine residues remain neutral with only one imidazole N atom (either Nδ or Nε) protonated. This pathway resembles some Mg^{2+}-dependent "substrate-assisted" kinase mechanisms.[18, 19] However, the DFT-calculated barrier (41.2 kcal/mol, Figure S12, Table S4) is rather high for this to be feasible, and shuttling by the phosphoryl group of a proton from Thr285 to the departing lipid would appear unfavourable.

If His395 is protonated, a suitable alternative reaction pathway (A), resembling previous proposals for phosphonate monoester hydrolase[17] or "base-assisted" kinase mechanisms,[18, 19] is found, with phosphate cleavage concerted with two proton transfers, from Thr285 to the Glu246 carboxylate, and from His395 to the dephosphorylated lipidic leaving group (Figure 3). The same path is obtained if His395 and His478 are both protonated (note that His466 coordinates Zn1 and cannot be protonated) with a free energy reaction barrier height of 10.2 kcal/mol (compared with 20.3 kcal/mol when only His395 is protonated) and a free energy of reaction of -11.5 kcal/mol (Figures 3, S12; Table S4). Reaction pathways A (with His395 and His478 neutral to permit zinc coordination) and B were also investigated using dizinc models based on the MCR-1 crystal structure (5LRM). In all cases the resulting energies were similarly unfavourable, with the lowest reaction barrier obtained unfeasibly high at 33 kcal/mol (Table S4). Single-point calculations identified only small variations in barrier height with dielectric constant (Table S5), confirming the size of the cluster model to be appropriate. These new DFT calculations thus support the contention that the first step of the MCR reaction is energetically feasible with only one Zn^{2+} ion, and moreover imply that PEA transfer to MCR Thr285 occurs by a concerted, rather than stepwise process. This mechanism likely requires protonation of at least His395, with a further reduction in barrier when His478 is also protonated. Our findings also accord with recent theoretical studies of phosphate ester hydrolysis[23] that identify "substrate-assisted" mechanisms as disfavoured for all but poor leaving groups.

Despite the wide distribution and clinical significance of MCR enzymes, the mechanism of PEA transfer remains poorly

understood, and is complicated by structural evidence for multiple metallated states. Our data demonstrate the MCR periplasmic catalytic domain to be a discrete structural entity that stably binds a single Zn^{2+} ion, as evidenced by rapid dissociation of a second Zn^{2+} equivalent during MD simulations. DFT calculations, as used to model a range of zinc metalloenzyme systems, including alkaline phosphatase (AP)[24], identify monometallic MCR-1 as supporting PEA transfer to Thr285 (the first step of lipid A modification) in a concerted mechanism involving Glu246 and His395 in proton transfer steps. Glu246 then adopts monodentate Zn1 coordination, as seen in MD simulations and crystal structures[7], and favoured by DFT models. Interaction of the unbound oxygen of MCR-1 Glu246 with Asn329 is reminiscent of interaction of Asp12 with Tyr105 in phosphonate monoester hydrolase[17] and Asp54 with Tyr205 in nucleotide pyrophosphatase/phosphodiesterase[25], respectively mono- and bimetallic enzymes that, like MCR, hydrolyse phosphate diester substrates. In AP, which favours phosphate monoesters[25], Asp51 (equivalent to MCR-1 Glu246) instead coordinates a Mg^{2+} ion absent from MCR-1 (Figure S13). Our proposed mechanism is then consistent with comparison of MCR with AP superfamily members with similar activities.

In summary, our data suggest a mechanism for PEA transfer to MCR Thr285 that explains the essentiality of His395 in the absence of a second zinc ion, distinguishes bacterial PEA transferases from multinuclear alkaline phosphatase, and is instead more reminiscent of those proposed for monometallic members of the wider AP superfamily. Scaffolds with zinc-binding functionality able to hydrogen bond to charged His395 then warrant evaluation as candidate inhibitors of this important antibiotic resistance mechanism.

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Conflicts of interest

There are no conflicts to declare.

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